Methods for Introgressing Resistance Alleles into Tomato

Technical Field of the Invention

The present invention relates to new and novel methods of introgressing resistance alleles into tomato (*Lycopersicon esculentum* L.). More specifically, the present invention relates to the introgression into the genome of tomato plants of an allele that encodes for resistance to tomato yellow leaf curl virus and an allele that encodes for resistance to root-knot nematodes. The methods involve selecting tomato plants wherein the allele that encodes for resistance to tomato yellow leaf curl virus and the allele that encodes for resistance to root-knot nematodes are present in the genome of a tomato plant in the coupling phase.

Background of the Present Invention

The goal of plant breeding is to develop new, unique and superior cultivars. Theoretically, a breeder can generate billions of different genetic combinations via crossing, selfing and selection. A breeder, however, has neither direct control of the location nor frequency of genetic recombination at the subcellular level. Therefore, two breeders will never develop the same line, or even very similar lines, having precisely the same traits. Descriptions of breeding methods that are commonly used for different traits and crops, as well as specifically for tomato, can be found in one of several reference books (i.e., Allard, R.W., *Principles of Plant Breeding* (1960); Simmonds, N.W., *Principles of Crop Improvement* (1979); Sneep, J. *et al.*, (1979) Tomato Breeding (p. 135-171) in: *Breeding of Vegetable Crops*, Mark J. Basset, (1986, editor), *The Tomato crop: a scientific basis for improvement*, by Atherton, J.G. & J. Rudich (editors), *Plant Breeding Perspectives* (1986); Fehr, *Principles of Cultivar Development—Theory and Technique* (1987).

Plant breeding begins with the analysis and definition of problems and weaknesses of the current varieties, the establishment of program goals, and definition of specific breeding objectives. The next step is the selection of germplasm that possesses traits to meet the program goals and the definition of the best breeding method to reach those goals. The objective is to combine in a single hybrid variety an improved combination of desirable traits from the parental germplasm. Important tomato

characteristics include higher yield, better fruit flavor, improved color and an extended life of the fruit. The latter characteristic is commonly referred to in fresh market cultivars as long shelf life. In processing cultivars used to make paste or ketchup, extended life in the field prior to harvest is referred to as holding ability. Other important traits that a breeder may include are resistance to pathogens, like viral and fungal diseases, insects, and tolerance to abiotic stress like drought and heat, along with characteristics related to seed yield of the seed parent lines to lower the cost of hybrid seed production.

The method chosen for breeding or selection depends on the mode of plant reproduction, the heritability of the trait(s) being integrated or improved, and the cultivar (i.e. variety) used commercially (i.e. F_1 hybrid or a pure breeding variety). The complexity of the inheritance influences the choice of breeding method. One simple method of identifying a superior plant is to observe its horticultural performance relative to other experimental plants or to a widely grown standard cultivar, or to observe its performance in hybrid combinations with other inbred lines. Breeder evaluations for the creation of new lines can be performed on single plants, or more often on more plants in small field plots, in multiple locations and during different seasons to provide a better estimate of its horticultural value. Proper testing and evaluation should detect any major faults and establish the level of superiority in horticultural value or improvement over current cultivars.

Breeding of commercial tomato hybrids requires the development of inbred parent lines. Because of inbreeding, these lines have a high degree of homozygosity throughout the genome. Desirable traits from two or more germplasm sources or gene pools are combined by breeders to develop said superior parent lines. One method of creating desirable inbreds or parent lines is by continuous self-fertilization and selection of the best breeding lines. Various methods, such as disease testing, biochemical evaluations, and DNA markers, can assist the breeder in the selection process. Subsequently, parent lines are combined in many different pair-wise combinations to identify the best hybrid varieties. Today the majority of commercial tomato varieties are hybrid varieties.

Once the parent lines that give the best hybrid combinations have been identified, the hybrid seed can be produced indefinitely, as long as the homogeneity and

the homozygosity (i.e. genetic purity) of the inbred parent lines is maintained. When different parent lines are crossed, the resulting progeny are called the first filial or F_1 generation. Hybrid seeds are F_1's. If F_1 plants are self-pollinated, the resulting progeny are called the second filial, or F_2 generation. A single-cross hybrid is produced when two inbred lines are crossed to produce the F_1 progeny. Hybrid varieties are valued for a number of reasons. First, hybrid seed can exhibit "hybrid vigor" (or heterosis) which can be manifested as an increased performance of a hybrid when compared with the mean of its inbred parents. Performance parameters can include increased yield, better germination rates, or higher seedling or plant vigor. Often, the hybrid vigor exhibited by F_1 hybrids is lost in the next generation (F_2). Second, hybrid seed allows for the production of a uniform product that exhibits a predictable performance and is better buffered against varying environmental conditions. Another benefit is that identical reproduction of the hybrid is impossible without access to the parental seed of the female and the male parents, thus providing the developer of the hybrid with biological protection on the commercial product (i.e., the F_1 hybrid).

Most importantly, another reason for developing and marketing of hybrid varieties is the fact that the characteristics of both parents are combined in the hybrid. For traits that exhibit a dominant inheritance, it is not required to combine all desirable characteristics in each of the hybrid parents. To combine all such characteristics into single breeding lines would require extensive breeding. By making hybrid combinations between two parental lines, a hybrid variety combines the characteristics of both parents in a single F_1 hybrid variety. Hybrid varieties are genetically homozygous at specific locations or loci if the genes responsible for the expression of a certain characteristic in both parent lines contain the same alleles, and heterozygous at other loci if corresponding genes in the parents contain different alleles. It is well known in the art that many favorable characteristics are based on dominant alleles. Because of dominance inheritance, a weakness in one of the parents (i.e. carrying a recessive allele) can be compensated in the hybrid by the contribution of the other parent that contains the dominant allele.

Certain traits, while commercially valuable, are undesirable when present in a homozygous condition. One such example is the *Mi-1* introgression that encodes for nematode resistance. The *Mi-1* allele that confers resistance to most races of

Meloidogyne incognita nematodes was introgressed into the cultivated tomato in the 1940's from *Lycopersicon peruvianum*, PI 128657, which is a wild relative of tomato (See Smith, *Proc. Amer. Soc. Hort. Sci.*, 44:413-416 (1944)). When the *Mi-1* allele is genetically fixed in a pure breeding line (*i.e.* an inbred line or a parent line) this line will not perform well horticulturally. This is particularly common for genes that originate from related or wild *Lycopersicon* species, such as *L. peruvianum* or *L. chilense.* These traits that cause defects are referred to as "genetic or linkage drag" and are well known to those skilled in the art.

Genetic drag can be the result of a pleiotropic effect of the introgressed gene itself. Such a gene could, for example, confer both a desirable phenotype like a disease resistance trait, and a horticulturally undesirable trait or defect, such as a stunted plant. In maize, for example, it is thought that the T-*urf13* mitochondrial gene results in a highly desirable phenotype called cytoplasmic male sterility. Unfortunately, this gene also confers a unique susceptibility to a fungal pathogen (Braun *et al.*, *Proc. Natl. Acad. Sci. USA* 86:4435-4439 (1989)). Pleiotropic examples, however, are uncommon. Far more often, other genes that are tightly linked genetically to the trait of interest cause the genetic drag from the wild species. While not wishing to be bound by any theory, it is believed that the genetic drag associated with the *Mi-1* introgression is the result of other genes linked to the *Mi* gene rather than a pleiotropic effect of the *Mi-1* gene itself. Tomato breeders have struggled to reduce this genetic drag because genetic recombination is known to be reduced in this region of the tomato genome (See Messeguer *et al.*, *Theor. Appl. Genet.* 82:529-536 (1991), Ho *et al.*, *Plant J.*, 2:971-982 (1992), Liharska *et al.*, *Genome* 39, 485-491 (1996) and Ganal *et al.*, *Theor. Appl. Genet.* 92:101-108 (1996)). Thus, although the *Mi-1* gene was first introgressed into cultivated tomato in the 1940's, it was not until the 1970's that the trait was established in commercial products.

As discussed briefly above, genetic drag can cause a number of undesirable horticultural traits or defects. One example of such a defect caused by genetic drag is reduction of fertility (other horticultural defects caused by genetic drag are described by J. Philouze & H. Laterrot in "Amélioration des Espèces Végétales Cultivées" by A. Gallais & H. Bannerot, INRA, pp. 379-391(1992)). Such defects are particularly strong if the environmental conditions are unfavorable. Moreover, these defects can be masked

or hidden when present in a heterozygous condition, such as in hybrid varieties. It is for this reason that alleles for disease resistance genes are often present in a heterozygous condition in commercial hybrid varieties, such as, but not limited to, varieties containing the *Mi-1* gene conferring resistance to nematodes (Laterrot, H., *INRA, OEPP/EPPO Bulletin* 3(1): 89-92 (1973). It is the experience of the inventors that varieties or lines homozygous for *Ty-1* gene, introgressed from *L. chilense* (and hence resistant to tomato yellow leaf curl virus) also exhibit horticultural defects due to genetic drag that is associated with the *Ty-1* gene.

Tomato is an important crop throughout the world. It has been estimated that there are 300,000 higher plant species on earth, but modern agriculture is based on less than 100 of these species. The cultivated tomato is a member of the *Solanaceae* family, which is the third most valuable crop family in the United States. Only the grasses and legumes are more valuable.

Modern tomato is a species that originated in the New World, and has been a part of many cultural diets for several centuries. *Solanum* species have a general adaptation to variable climatic growing conditions. The cultivated tomato (*Lycopersicon esculentum*) is adapted to warm summer growing conditions, but can also be grown in heated greenhouses under winter conditions. The introduction of hybrid cultivars in the 1950's provided benefits like increased yield, better holding ability, adaptation to expanded growing seasons through the use of protected cultivation and improved disease resistance.

All varieties in the species *L. esculentum* are self-pollinating. Most other species in the genus *Lycopersicon* are self-incompatible, which means that they require cross-pollination in order to set seeds. Insects, primarily honey- or bumble-bees perform such cross-pollination. Tomato, like most other *Lycopersicon* species, exhibits large inherent variability that can be observed in the wide variety of fruit sizes, shapes and colors available in the marketplace. A consequence of pollination methods is that *L. esculentum* varieties lend themselves to a high degree of inbreeding, and that wild species have a high degree of heterozygosity. Indeed, even within wild accessions, like *L. chilense* accession LA1969, it is not uncommon to find a heterogenous population of

alleles. It is this inherent heterogeneity that provides breeders with a reservoir of novel traits for adaptation through classical breeding to produce new commercial cultivars.

Like other crops, tomatoes are susceptible to a variety of different pathogens. By way of example, Geminiviruses cause serious disease problems in a number of regions in the world, including the Mediterranean basin, Near- and Middle East, North America, Asia and parts of Africa. Numerous Geminiviruses are known in the art. One example of a Geminivirus is tomato yellow leaf curl virus (TYLCV). Another serious pathogen of tomato are root-knot nematodes, which can cause serious disease problems in most regions of the world.

TYLCV is transmitted to the tomato by the whitefly, *Bemisia tabaci*. Infection by tomato yellow leaf curl virus is known to cause considerable damage to tomato crops, with losses of up to 50 to 60% (See Allex *et al. PHM Revue Horticol N.*, 350:13-17 (1994)). Characteristic disease symptoms in tomato include the stunted appearance of the plant, leaf curling and yellowing, a bush-like appearance due to the shortening of the internodes and arrest of floral growth.

Plant breeders have sought sources of resistance to TYLCV within tomato. Early efforts to identify sources of resistance to TYLCV within *L. esculentum* only revealed the existence of some intermediately resistant sources in cultivated tomatoes (See Francisco J. Morales, "*Breeding for Resistance to Whitefly-Transmitted Geminiviruses*", Tomato Breeders Roundtable (2001)). However, in 1966, Cohen and Nitzany (See Cohen and Nitzany, *Phytopathology*, 56:1127-1131 (1966)) observed that some wild species related to tomato, such as *L. pimpinellifolium* and *L. peruvianum*, possessed a higher level of resistance to TYLCV, although they were not immune. In 1994, a TYLCV resistance allele at the *Ty* locus in *L. chilense*, designated as *Ty-1,* was described (See Zamir *et al. Theor. Appl. Genet.*, 88:141-146 (1994)). This resistance was present in the wild tomato accession LA 1969. LA 1969 is publicly available from the C.M. Rick Tomato Genetics Resource Center, Department of Vegetable Crops, University of California, One Shields Avenue, Davis, CA 95616.

In addition to being infected by Geminiviruses, tomatoes are also infected by a variety of pests, such as nematodes. By way of example, root-knot nematodes, such as

those belonging to the species *Meloidogyne*, are particularly problematic and cause problems in typically all growing areas. There are more than fifty (50) species of root-knot nematodes known in the art. The three most commonly occurring and most damaging species of root-knot nematodes are *M. arenaria, M. incognita* and *M. javanica.*

Nematodes are tiny, worm-like organisms that are found in almost every soil environment on earth. Root-knot nematodes are so named because when they infect certain plant species, they cause malformation of the roots. Root-knot nematodes cause lumps or galls all over the roots. These lumps or galls range in size from 1 to 10 mm in diameter. The nematodes reproduce themselves in these galls. These malformed roots are compromised in their ability to supply water and nutrients to the plant. Root-knot nematodes typically do produce indirect symptoms above ground. For example, infected plants typically lack plant vigor and often show symptoms of stunting, wilting or chlorosis (i.e. yellowing). Typically, the nematode population does not build up until later in the growing season. It is at this time that the plants begin to wilt and consequently fruit set and development is reduced.

In 1944, resistance to root-knot nematodes of the *Meloidogyne* species was identified in the wild tomato species, *L. peruvianum* PI 128657 (See Smith, *Proc. Amer. Soc. Hort. Sci.*, 44:413-416 (1944)). This gene, designated as *Mi*-1, confers resistance to three species of nematodes (*M. arenaria, M. incognita* and *M. javanica)*. While *Mi-1* is a very effective nematode resistance gene, it presents problems when used in breeding, because of the linkage between the *Mi* locus and neighboring genes on chromosome 6 that encode for unfavorable characteristics and are coinherited with the *Mi-1* allele. More specifically, most of the wild relatives of the modern tomato, like *L. peruvianum*, are wild growing, have small, poor tasting green fruit, along with a variety of other poor horticultural traits. When a breeder attempts to introduce a desirable trait from a wild species into the modern tomato, such as the *Mi-1* gene from *L. peruvianum*, linked genes that result in poor horticultural performance are also introgressed. Because of this genetic drag, open pollinated and homozygous tomato varieties containing the *Mi-1* allele were not be sold commercially until the middle 1970's after part of the coinherited alleles had been disassociated from the *Mi* locus through the process of repeated random recombination. Hybrid varieties overcame most of the genetic drag by masking the effects by having a heterozygous genotype at the *Mi* locus. Even today, the

majority of modern commercial nematode resistant hybrid tomato varieties are heterozygous for the *Mi-1* allele. The wild type allele *Mi*$^{+}$ from cultivated tomato, with its associated favorable genes linked on the same chromosome, is preferred to be present in the hybrid to compensate for the genetic drag associated with the *Mi-1* allele from *L. peruvianum*.

There is a need in the art for new tomato varieties that exhibit combined resistance to TYLCV and root-knot nematodes. It is known in the art that both the *Mi* and *Ty* loci are located in the centromeric region of chromosome 6 and are tightly linked genetically. The development of inbred lines containing both the *Ty-1* and *Mi-1* alleles as a single inheritable unit has proven to be complicated. One problem has been the repression of recombination events near the *Mi* and *Ty* loci located on chromosome 6 (See Messeguer *et al.*, *Theor. Appl. Genet.* 82:529-536 (1991), Ho *et al.*, *Plant J.*, 2:971-982 (1992), Liharska *et al.*, *Genome* 39, 485-491 (1996) and Ganal *et al.*, *Theor. Appl. Genet.*, 92:101-108 (1996)).

Another problem has been the discovery of an allele called "*Mi-J*" at the *Mi* locus in *L. chilense* that encodes for an intermediate level of resistance to the root-knot nematode, *M. incognita*. The intermediate level of resistance encoded by the *Mi-J* allele is lower than the resistance encoded by the *Mi- 1* allele from *L. peruvianum*. The *Mi-J* allele was discovered in inbred tomato line FDR 16-2045 that is described in U.S. Patent No. 6,414,226. This patent is owned by the assignee of the present invention. FDR 16-2045 contains the *Ty-1* allele and was initially believed also to contain the *Mi-1* allele, because of its resistance to root-knot nematodes. However, further pathology testing demonstrated that this line was found to provide a lower level of resistance to the root-knot nematode *Meloidogyne incognita* than lines containing the *Mi-1* allele. This result was unexpected because the *Mi-1* allele is known to provide complete resistance. Based on this knowledge, a plant breeder would have expected FDR 16-2045 to be either completely resistant or susceptible to *M. incognita*. Further investigation into FDR 16-2045 revealed that this line did not contain the *Mi-1* gene from *L. peruvianum* but instead contained a gene from *L. chilense* at the *Mi locus* that confers an intermediate level of resistance to the root-knot nematode, *M. incognita*. This gene from *L. chilense* that confers an intermediate level of resistance is referred to as the "*Mi-J* allele". The *Mi-J* allele present at the *Mi* locus was introgressed along with the *Ty-1* allele from *L.*

chilense and was found to differ from the *Mi-1* allele of *L. peruvianum*. The *Mi-J* allele is located near the *Ty-1* locus on chromosome 6 and, as mentioned previously, the level of resistance conferred by the *Mi-J* allele is not as efficacious as the resistance conferred by the *Mi-1* allele. This finding of another allele at the *Mi*-locus with different pathogenic efficacy provides additional evidence that wild-species are highly heterogenous, and thus contain numerous mutant alleles.

Based on the teachings described in U. S. Patent No. 6,414,226, a plant breeder could create an inbred line, like FDR 16-2045 with the *Ty-1* allele and the *Mi-J* allele, and then produce hybrids with resistance to both TYLCV and nematodes. If the breeder crossed an inbred like FDR 16-2045 with an inbred having the wild type alleles Ty^+, Mi^+, the breeder could mask the genetic drag associated with the introgression, while maintaining a level of complete resistance to TYLCV, but, only intermediate resistance to nematodes due to the presence of the *Mi-J* allele.

However, for a plant breeder to improve on this combination in order to obtain a higher level of nematode resistance is difficult. Using the genetic resources available to those skilled in the art, a plant breeder could employ two (2) different strategies. First, the breeder could cross an inbred line such as FDR 16-2045 with an inbred line that contains the *Mi-1* allele to create a hybrid having a level of complete resistance to both TYLCV and to nematodes. This first strategy would create hybrids having a high level of nematode resistance, but with the associated negative effects of the genetic drag. Second, a breeder could cross an inbred line like FDR 16-2045 with another inbred line containing both the wild type alleles at the Ty^+ and Mi^+ loci. This second strategy, apart from TYLCV resistance, would create intermediately nematode resistant hybrids, without the effects of genetic drag. Both strategies would require breeders to create and maintain separate germplasm pools to make these hybrids. Most problematic, however, is that neither of these strategies would facilitate the breeding of complete nematode resistant varieties without the negative effects of genetic drag. Thereupon, one object of the present invention is to provide such a method of breeding that eliminates these problems.

It would be beneficial for plant breeders to have the ability to create hybrids having the superior resistance alleles for both Geminivirus resistance (such as, but not

limited to, the *Ty-1* allele and polymorphic variants thereof) and nematode resistance (such as but not limited to, the *Mi-1* allele and polymorphic variants thereof) as a single co-inheritable unit while retaining the ability to mask genetic drag associated with each allele that has originated from a wild species (such as, but not limited to, *Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii, Lycopersicon peruvianum, Lycopersicon chilense* and *Solanum lycopersicoides*). Thereupon, it is another object of the present invention to provide improved and more flexible methods of combining TYLCV resistance and root-knot resistance as a single coinheritable unit into tomato germplasm using a combination of molecular biology, plant pathology and traditional breeding techniques. This invention provides methods to create the best available alleles for resistance to TYLCV and nematodes in hybrid combination in a tomato plant, while retaining the ability to mask the effects of genetic drag. This invention not only contemplates combining the *Ty-1* and *Mi-1* alleles as a single coinheritable unit *in cis* for use in breeding, but further contemplates combining *in cis* any new alleles (i.e. polymorphic variants) that are identified or discovered at the *Ty* and/or *Mi* loci that have improved pathogen resistance efficacy.

Brief Description of the Figures

Figure 1 depicts a cross between two parental breeding lines P1 and P2. The P1 line has the homozygous genotype *Ty-1*, *Mi-J* and the P2 line has a homozygous genotype Ty^+, *Mi-1*. The F_1 is heterozygous at the *Ty* and *Mi* loci, but is hampered by the genetic drag associated with the *Mi-J* and *Mi-1* alleles. Gametes from the F_1 would be almost exclusively be made up of the combinations *Ty-1 Mi-J* or Ty^+ *Mi-1*. During meiosis, however, there could be a rare recombination between the *Ty* and *Mi* loci to create the gametes with the genotypes Ty^+ *Mi-J* and/or *Ty-1 Mi-1*. The genotypic structure of the F_2 generation would contain a majority of plants having each of the parental genotypes or the genotype of the F_1. Depending on the size of the F_2 population, the F_2 may also contain the rare recombinants. The first recombinant combination, Ty^+ *Mi-J* has the susceptible allele for TYLCV resistance, and the *Mi-J* allele giving an intermediate level of resistance for nematode resistance. The second recombinant combination, *Ty-1 Mi-1* is an event of interest, because it contains both the resistant alleles in combination. When alleles are in this combination, they are referred to as being in the "coupling phase" or "*in cis*." However, it is very unlikely that this

desired combination would be genetically fixed in the F_2. Moreover, the identification and selection of plants containing the *Ty-1* and the *Mi-1* alleles *in cis*, using normal methods of breeding, could not be accomplished because of the inability to distinguish between the *Mi-1* and *Mi-J* alleles at the single plant level using traditional plant pathology testing methods. Fixation of this novel combination of traits would be typically achieved by selfing said specific F_2 individual, and selecting homozygous plants with the genotype *Ty-1 Mi-1* from the third filial generation (or F_3).

Figure 2 shows the digestion of amplified DNA created by the assay described in Example #2 from plants that contain either the resistant *Ty-1* allele or the susceptible Ty^+ allele. DNA size standards, available from Invitrogen, Carlsbad, CA are shown in the far left lane. Lane A shows the digestion products of amplified DNA from a plant that contains the resistant *Ty-1* allele; with this genotype, two fragments of 303 and 96 base pairs are produced. Lane B shows the digestion of amplified DNA from a plant that contains a susceptible Ty^+ allele; with this genotype a single fragment of 398 base pairs is produced.

Figure 3 shows the digestion of amplified DNA created by the assay described in Example #3 from plants that contain either the *Mi-1* allele or *Mi-J* allele or the Mi^+ allele. DNA size standards, available from Invitrogen, Carlsbad, CA are shown in the far left lane. Lane A shows the digestion products of the amplified DNA from a plant that contains either the *Mi-1* allele or *Mi-J* allele; with these genotypes, two fragments of approximately 145 and 450 base pairs are produced. Lane B shows the digestion of amplified DNA from a plant that contains an Mi^+ allele; with this genotype a single fragment of 595 base pairs is produced.

Figure 4 shows the digestion of amplified DNA created by the assay described in Example #4 from plants that contain either the *Mi-1* allele or *Mi-J* allele or the Mi^+ allele. DNA size standards, available from Invitrogen, Carlsbad, CA are shown in the far left lane. Lane A shows the digestion of amplified DNA from a plant that contains either a *Mi-1* allele from *L. peruvianum* or the Mi^+ wild type allele from *L. esculentum;* with these genotypes a single fragment of 282 base pairs is produced. Lane B shows the digestion products of amplified DNA from a plant that contains the *Mi-J* allele; with this genotype two fragments of approximately 124 and 158 base pairs are produced.

Figure 5 shows a comparison of the polynucleotide sequences of the *Ty+* (SEQ ID NO.: 11) and *Ty-1* (SEQ ID NO.: 10) alleles and the identification in the shaded boxes of twenty-one single nucleotide polymorphisms between the polynucleotide sequences. The circle identifies two (2) adjacent polymorphisms at base pairs 97-98 in the *Ty-1* sequence and 96-97 in the *Ty+* sequence.

Figure 6 shows a comparison of the polynucleotide sequences of the *Mi+*, *Mi-1*, and *Mi-J* alleles and the identification in the shaded boxes of nineteen (19) single nucleotide polymorphisms between the polynucleotide sequences. The circles identify polymorphisms at base pairs 603 and 754.

Figure 7 shows the polynucleotide sequences at the marker loci of the *Mi+* (SEQ ID NO.: 7), *Mi-1* (SEQ ID NO.: 8) and *Mi-J* (SEQ ID NO.: 9) alleles and the *Ty-1* (SEQ ID NO.: 10) and *Ty+* (SEQ ID NO.: 11) alleles.

Figure 8 shows the normalization of the data provided in Table 1 in Example 7 using pathology scoring. More specifically, the results in Table 1 and Figure 8 demonstrate the efficacy between the *Mi-J* and *Mi-1* alleles, particularly when these alleles are present in the heterozygous condition in a hybrid plant.

Summary of the Present Invention

In one embodiment, the present invention relates to a method of making a *Lycopersicon esculentum* plant that contains within its genome an allele that encodes for resistance to tomato yellow leaf curl virus (i.e. such as a *Ty-1* allele or a polymorphic variant thereof) in the coupling phase (or *in cis*) with an allele that encodes for resistance to root-knot nematodes (i.e such as a *Mi-1* allele or a polymorphic variant thereof). A plant containing these alleles is resistant to both tomato yellow leaf curl virus and root-knot nematodes. The method involves the following steps: (a) identifying an allele in a species of tomato that encodes for resistance to tomato yellow leaf curl virus; (b) comparing a polynucleotide sequence of the allele identified in step a) with at least one polynucleotide sequence selected from the group consisting of SEQ ID NO.: 10 and SEQ ID NO.: 11 and identifying at least one polymorphism (such as, but not limited to, a single nucleotide polymorphism) between the polynucleotide sequences; (c) designing a

molecular test that can discriminate/distinguish a polymorphism identified in step (b); (d) identifying an allele in a species of tomato that encodes for resistance to root-knot nematodes; wherein said allele is from a different species of tomato than the allele identified in step a) that encodes for resistance to tomato yellow leaf curl virus; (e) comparing a polynucleotide sequence of the allele identified in step d) with at least one polynucleotide sequence selected from the group consisting of SEQ ID NO.: 7, SEQ ID NO.: 8 and SEQ ID NO.: 9 and identifying at least one polymorphism (such as, but not limited to, a single nucleotide polymorphism) between the polynucleotide sequences; (f) designing a molecular test that can discriminate/distinguish a polymorphism identified in step (e); and (g) introgressing into the genome of a *Lycopersicon esculentum* the allele identified in step a) and the allele identified in step (d) in the coupling phase using traditional breeding techniques, plant pathology testing and marker-assisted selection, wherein the marker-assisted selection employs the molecular tests designed in steps c) and f) (which includes, but is not limited to, the design of one or more primer pairs). The at least one polymorphism can result in a difference in a restriction endonuclease recognition site in the allele identified in step a) when compared to SEQ ID NO.: 10 and/or SEQ ID NO.: 11 and in a restriction endonuclease recognition site in the allele identified in step d) when compared to SEQ ID NO.: 7, SEQ ID NO.: 8 and/or SEQ ID NO.:9.

In yet another embodiment, the present invention relates to a *Lycopersicon esculentum* plant produced by the above-described method. The *Lycopersicon esculentum* plant produced by the above-described method can be an inbred or hybrid plant. The present invention also contemplates parts of the *Lycopersicon esculentum* plant produced by the above-described method, including, but not limited to, cells, cell tissue cultures, callus (calli), cell clumps, embryos, leaves, petals, stems, roots, root tips, shoots, protoplasts, somatic embryos, anthers, petioles, ovules, pollen, styles, stamens and seed.

In yet another embodiment, the present invention relates to a method for producing a *Lycopersicon esculentum* plant (such as an inbred or hybrid plant) in a tomato breeding program. The method involves the steps of (a) obtaining the *Lycopersicon esculentum* plant or its parts or a descendant of the *Lycopersicon esculentum* plant or its parts described above as a source of breeding material; and (b)

employing the *Lycopersicon esculentum* plant or part thereof obtained in step a) as a source of plant breeding material in a plant breeding program using plant breeding techniques to produce a *Lycopersicon esculentum* plant. The plant breeding techniques that can be employed in said method include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, mutagenesis, transformation or combinations of these techniques or portions of these techniques. The present invention further contemplates *Lycopersicon esculentum* plants (such as inbreds and hybrids) produced by this method as well as parts of said *Lycopersicon esculentum* plants (parts include, but are not limited to, cells, cell tissue cultures, callus (calli), cell clumps, embryos, leaves, petals, stems, roots, root tips, shoots, protoplasts, somatic embryos, anthers, petioles, ovules, pollen, styles, stamens and seed).

In another embodiment, the present invention relates to a method of making a *Lycopersicon esculentum* plant that contains within its genome at least one tomato yellow leaf curl virus resistance allele designated as *Ty-1* or a polymorphic variant thereof in the coupling phase with at least one allele that encodes for resistance to root-knot nematodes designated *Mi-1* or a polymorphic variant thereof. A *Lycopersicon esculentum* plant produced by this method and containing the *Ty-1* and *Mi-1* alleles in coupling phase is resistant to both tomato yellow leaf curl virus and root-knot nematodes. The method involves introgressing into the genome of a *Lycopersicon esculentum* a *Ty-1* allele or polymorphic variant thereof that encodes for tomato yellow leaf curl virus resistance and a *Mi-1* allele or a polymorphic variant thereof that encodes for resistance to root-knot nematodes in the coupling phase. The introgression is achieved using marker-assisted selection. The marker-assisted selection testing employs at least one of the following primer pairs:

5' TAATCC GTCGTTACCTCTCC TT 3' (SEQ ID NO.: 1) and 5' CGGATGACTTCAATAGCAATGA 3' (SEQ ID NO.: 2);

5' AACCGTGGAC TTTGCTTTGA CT 3' (SEQ ID NO.: 3) and 5' TAAGAACAGG GACTCAGAGG ATGA 3' (SEQ ID NO.: 4);

5' CTACGGAGGATGCAAATAGA A 3' (SEQ ID NO.: 5) and 5' AATCATTATT GTCACACTTCCCC 3' (SEQ ID NO.: 6), or

variants of any of SEQ ID NOS: 1-6.

The *Ty-1* allele and *Mi-1* allele or polymorphic variants thereof used in the above-described method are each derived from a different species of tomato, such as, but not limited to, *Lycopersicon esculentum, Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii, Lycopersicon peruvianum, Lycopersicon chilense or Solanum lycopersicoides.*

In yet another embodiment, the present invention relates to a *Lycopersicon esculentum* plant produced by the above-described method. The *Lycopersicon esculentum* plant produced by the above-described method can be an inbred or hybrid plant. The present invention also contemplates parts of the *Lycopersicon esculentum* plant produced by the above-described method, including, but not limited to, cells, cell tissue cultures, callus (calli), cell clumps, embryos, leaves, petals, stems, roots, root tips, shoots, protoplasts, somatic embryos, anthers, petioles, ovules, pollen, styles, stamens and seed.

In yet another embodiment, the present invention relates to a method for producing a *Lycopersicon esculentum* plant (such as an inbred or hybrid) a tomato breeding program. The method involves the steps of (a) obtaining the *Lycopersicon esculentum* plant or its parts or a descendant of the *Lycopersicon esculentum* plant or its parts described above as a source of breeding material; and (b) employing the *Lycopersicon esculentum* plant or part thereof obtained in step a) as a source of plant breeding material in a plant breeding program using plant breeding techniques to produce a *Lycopersicon esculentum* plant. The plant breeding techniques that can be employed in said method include, but are not limited to, recurrent selection, backcrossing, pedigree breeding, mutagenesis, transformation or combinations of these techniques or portions of these techniques. The present invention further contemplates *Lycopersicon esculentum* plants (such as inbreds and hybrids) produced by this method as well as parts of said *Lycopersicon esculentum* plants (parts include, but are not limited to, cells, cell tissue cultures, callus (calli), cell clumps, embryos, leaves, petals, stems, roots, root tips, shoots, protoplasts, somatic embryos, anthers, petioles, ovules, pollen, styles, stamens and seed).

In yet a further embodiment, the present invention relates to a *Lycopersicon esculentum* plant that comprises within its genome a tomato yellow leaf curl virus resistance allele designated as *Ty-1* in coupling phase with an allele for resistance to root-knot nematodes designated as *Mi-1*. Such a plant is resistant to both tomato yellow leaf curl virus and root-knot nematodes. The *Ty-1* allele and *Mi-1* allele are present in the genome of the plant in the coupling phase on chromosome 6. The *Ty-1* allele and the *Mi-1* allele introgressed into the genome of the *Lycopersicon esculentum* plant are each derived from a different species of tomato, such as, but not limited to, *Lycopersicon esculentum, Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii, Lycopersicon peruvianum, Lycopersicon chilense or Solanum lycopersicoides*. The above-described *Lycopersicon esculentum* plant can be an inbred or hybrid plant or a plant part of either of such plants. Parts of such a plant include, but are not limited to, cells, cell tissue cultures, callus (calli), cell clumps, embryos, leaves, petals, stems, roots, root tips, shoots, protoplasts, somatic embryos, anthers, petioles, ovules, pollen, styles, stamens and seed.

Detailed Description of the Present Invention

Definitions

As used herein, the term "allele(s)" means any of one or more alternative forms of a gene at a particular locus, all of which alleles relate to one trait or characteristic at a specific locus. In a diploid cell of an organism, alleles of a given gene are located at a specific location, or locus (loci plural) on a chromosome. One allele is present on each pair of homologous chromosomes.

As used herein, the term "in the coupling phase" or "in *cis*" refers to a genetic condition in which the alleles of two different loci occur together linked on the same (homologous) chromosome. When this occurs, their 'phase' is said to be "in coupling". For example, when the alleles *Ty-1* and *Mi-1* are located on the same chromosome homologue, these alleles are "in the coupling phase". In contrast, if the alleles Ty^+ and *Mi-1* are located on different homologous chromosomes of a homologous pair, they are said to be "in the repulsion phase", or "in *trans*". Figure 1 provides an illustration of the

Ty-1 and *Mi-1* alleles in "repulsion phase" in P1 and P2 and in "coupling phase" in the F_2 progeny after selfing of an F_1 hybrid plant.

As used herein, the term "heterozygous" means a genetic condition existing when two different alleles reside at a specific locus, but are positioned individually on corresponding pairs of homologous chromosomes in the cell of a diploid organism.

As used herein, the term "homozygous" means a genetic condition existing when two identical alleles reside at a specific locus, but are positioned individually corresponding pairs of homologous chromosomes in the cell of a diploid organism.

As used herein, the term "hybrid" means any offspring of a cross between two genetically unlike individuals (See Rieger, R., A Michaelis and M.M. Green, 1968, *A Glossary of Genetics and Cytogenetics*, Springer-Verlag, N.Y.).

As used herein the term "homologues" means the members of a chromosome pair.

As used herein, the term "inbred" means a highly inbred, and thus substantially homozygous and pure breeding individual plant, line or variety.

As used herein, the term "introgressed" means the entry or introduction of a region of a chromosome from one plant species into another. As used herein, the term "introgressing" means entering or introducing a region of a chromosome from one plant species into another.

As used herein, the term "introgression" means the actual DNA fragment introduced or transferred from a related plant species into a cultivated species.

As used herein, the term "locus" (loci plural) means a specific place or places or a site on a chromosome where a gene or genetic marker is found.

As used herein, the term "plant" includes plant cells, plant protoplasts, plant cell tissue cultures from which tomato plants can be regenerated, plant calli, plant cell

clumps, and plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, leaves, seeds, roots, root tips and the like.

As used herein, the term "polymorphic variant" refers to variation in the sequence of nucleotides between alleles.

As used herein, the term "resistance", "complete resistance", "a high level of resistance" or "highly resistant" refers to the ability of a plant variety to limit or restrict the growth and development of a given pest or pathogen and/or the damage that such a pest or pathogen causes throughout the whole or part of the growing cycle (such as, but not limited to a virus, fungus or pest), when compared to susceptible varieties under similar environmental conditions and pest/pathogen pressure. Resistant varieties may exhibit some symptoms when specific pest or pathogen pressure is severe.

Varieties with an "intermediate level of resistance" or which are "intermediately resistant" refers to the ability of a plant variety to limit or restrict the growth and development of a given pest or pathogen and/or damage that such a pest or pathogen causes; however, such a plant variety may exhibit a greater range of symptoms compared to resistant varieties when grown under similar environmental conditions of moderate to severe pathogen pressure. Plant varieties that exhibit an intermediate level of resistance or are intermediately resistant show less severe symptoms or damage than susceptible plant varieties when grown under similar environmental conditions and/or pest pressure.

As used herein, the term "root-knot nematodes" refers to nematodes of the *Meloidogyne* genus, including but not limited to the species *M. arenaria, M. incognita* and *M. javanica*.

As used herein the term "susceptible", "susceptibility" or when a plant variety is "susceptible" refers to the inability of a plant variety to limit or restrict the growth and development of a given pest or pathogen and/or the damage that such a pest or pathogen causes in said plant.

As used herein, the term "variants thereof" or "variants of any of SEQ ID NOS.: 1-6" refers to a polynucleotide sequence having one or more nucleotides deleted (deletion variants) from said polynucleotide sequence or having one or more nucleotides substituted with other nucleotides or one or more nucleotides inserted into said polynucleotide sequence (insertional variants).

As used herein, the term "variety" or "cultivar" means a plant grouping within a single botanical taxon of the lowest known rank, which grouping can be defined by the expression of the characteristics resulting from a given genotype or combination of genotypes, can be distinguished from another plant grouping by the expression of at least one of the said characteristics and which is considered as a single unit with regard to its suitability for being propagated unchanged.

As used herein, the term "wild type", means the naturally occurring allele found within *L. esculentum*. At the nematode resistance locus *Mi,* and the TYLCV locus *Ty*, the wild type alleles from *L. esculentum* encode for a susceptibility to these pathogens.

Sequence Listing

The present application also contains eleven (11) nucleic acid sequences (SEQ ID NOS.: 1 to 11). For the nucleic acid sequences, the base pairs are represented by the following base codes:

Symbol	Meaning
A	adenine
C	cytosine
G	guanine
T	thymine
N	adenine or cytosine or guanine or thymine

Description of the Invention

The present invention relates to tomato plants (*Lycopersicon esculentum L.*) that are resistant to both tomato yellow leaf curl virus (TYLCV) and root-knot nematodes. More specifically, the present invention relates to the introgression into tomato germplasm (*Lycopersicon esculentum L.*) of an allele that encodes for resistance to TYLCV in the coupling phase (also known as *in cis*) with an allele that encodes for resistance to root-knot nematodes. The allele that encodes for resistance to TYLCV and

the allele that encodes for resistance to root-knot nematodes are each preferably derived from different germplasm sources (i.e., different species of tomato), such as, but not limited to, *Lycopersicon esculentum, Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii, Lycopersicon peruvianum, Lycopersicon chilense* or *Solanum lycopersicoides.* The introgression is performed in such a manner that an allele that encodes for resistance to TYLCV and an allele that encodes for resistance to root-knot nematodes are integrated into the genome of tomato on one chromosome in the coupling phase and are inherited from one generation to the next as a single Mendelian unit. Such introgression is achieved using a combination of molecular biology, plant pathology and traditional breeding techniques. More specifically, the present invention uses molecular biology techniques to discriminate between different alleles at certain loci (i.e. the *Ty* and *Mi* loci) to combine the desirable alleles for TYLCV resistance and root-knot nematode resistance into the genome of cultivated tomato on one chromosome as a coinheritable unit. Prior to the present invention, these two alleles could be present in a single plant, but only when located on different chromosomes of a chromosome pair (i.e. in repulsion phase, or *in trans*). The present invention facilitates the breeding of tomato hybrids with multiple resistance to both TYLCV and root-knot nematodes while enhancing the plant breeder's ability to mask the genetic drag that is typically associated with these traits.

By way of example, but not of limitation, the present invention provides for the introgression into tomato germplasm (*Lycopersicon esculentum L.*) of the *Ty-1* allele from *L. chilense* that encodes for resistance to TYLCV at the *Ty* locus and the *Mi-1* allele from *L. peruvianum* that encodes for resistance to root-knot nematodes at the *Mi* locus. One skilled in the art would recognize that the introgression of *Ty-1* and *Mi-1* alleles and polymorphic variants of these alleles from sources other than *L. chilense* and *L. peruvianum* (such as, but not limited to, *Lycopersicon esculentum, Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii* and *Solanum lycopersicoides*) is also contemplated within the scope of the present invention.

The methods of the present invention provide a number of benefits. First, these methods allow for the creation of pure breeding tomato lines, such as, but not limited to, tomato breeding lines that contain both the *Ty-1* allele and the *Mi-1* allele in homozygous condition on chromosome 6 as a coinheritable unit. Most importantly, these lines exhibit a much greater level of resistance to root-knot nematodes than tomato plants that are known in the art and that contain the *Mi-J* allele in combination with the *Ty-1* allele. As discussed previously, tomato plants that contain the *Mi-J* allele, such as tomato inbred line FDR 16-2045 (described in U.S. Patent No. 6,414,226), exhibit only an intermediate level of resistance to root-knot nematodes.

Tomato lines that are homozygous for alleles that encode for resistance to TYLCV and root-knot nematodes, such as, but not limited to, *Ty-1* and *Mi-1*, allow a breeder to make a hybrid tomato variety that contains these alleles (i.e. the *Ty-1* allele and the *Mi-1* allele) on one chromosome, and the susceptible wild type alleles on the other homologous chromosome. This facilitates more flexibility in the breeding program and allows for compensation of the unfavorable effects associated with the introgressed genes in the hybrid (i.e. genetic or linkage drag). Previously, in order to obtain resistance to TYLCV and to root-knot nematodes, breeders had to cross two lines, each line containing resistance to only one of these diseases. In the latter strategy, the ability of the breeder to mask genetic drag, especially the drag associated with the *Mi-1* allele, was limited.

As mentioned briefly previously, the present invention uses a combination of molecular biology, plant pathology and traditional breeding techniques. The molecular biology techniques used in the present invention involve certain marker-assisted selection assays that employ nucleic acid primers, which will be discussed in more detail below. The present invention not only contemplates the specific assays disclosed herein which involve the *Ty-1* and *Mi-1* alleles, but any assays that can be used to introgress into tomato any allele that encodes for resistance to TYLCV in the coupling phase with any allele that encodes for resistance to root-knot nematodes. For example, the present invention contemplates introgressing into tomato any polymorphic variants (i.e. mutant alleles) of the *Ty-1* and/or *Mi-1* alleles. Moreover, although the introgression of an allele that encodes for resistance to TYLCV and an allele that encodes for resistance to root-knot nematodes in the coupling phase must be performed one at a

time, the present invention contemplates the introgression of multiple disease resistance alleles in the coupling phase at each of the *Ty* and *Mi* loci.

In order to perform the marker-assisted selection in the methods of the present invention, the subject tomato plants or plant parts are first subjected to DNA extraction, the techniques of which are known in the art (See Hnetkovsky *et al.*, *Crop Sci.,* 36(2): 393-400 (1996)). Once the extraction is complete, a molecular assay can be performed, including, but not limited to, a cleaved amplified polymorphic sequence (CAPS) assay, the techniques of which are well known in the art (See Akopyanz *et al.*, *Nucleic Acid Research,* 20:6221-6225 (1992) and Konieczny & Ausubel, *The Plant Journal*, 4:403-410 (1993)). A CAPS assay involves amplifying the locus by PCR followed by digestion with restriction enzymes. Polymorphisms between the different alleles (such as, but not limited to, the *Mi+, Mi-J* and/or *Mi-1* alleles) can result in different sized restriction fragments.

Nucleic acid primers and enzymes are employed in these assays in order to identify which alleles are present at the *Ty* and/or *Mi* loci in the genome of a tomato plant, and, if said alleles are present, whether the alleles are present in a homozygous or heterozygous condition. The information obtained from both loci is used to identify those plants that have specific allelic combinations at the *Ty* and *Mi* loci in the coupling phase (i.e, *in cis*).

The primers used in the methods of the present invention are DNA polynucleotides that can be purchased from a commercially available source or synthesized using techniques which are known to those skilled in the art, including, but not limited to, conventional nucleotide phosphoramidite chemistry, using instruments available from Applied Biosystems, Inc. (Foster City, California). Moreover, when desired, the primers of the present invention can be modified for use in other marker-assisted selection assays, such as, but not limited to, the TaqMan® assay from Applied Biosystems, Foster City, CA, using techniques known in the art, including, but not limited to those described in U.S. Patent Nos. 5,464,746, 5,424,414 and 4,948,882.

To create a marker-assisted selection test, one skilled in the art begins by comparing the DNA sequence from the donor source (i.e. the germplasm containing the

disease resistance trait) with the corresponding DNA sequence from the recipient source (i.e. the germplasm containing the susceptible alleles for the specific pathogen). Alternatively, a sequence comparison between DNAs from the donor and recipient can be performed at corresponding positions in the genome that are tightly linked genetically to the trait of interest. For the *Ty* and *Mi* loci, identification of polymorphisms near these traits are known in the art (See, Zamir *et al.*, *Theor. Appl. Genet.* 88:141 (1994) and Williamson *et al.*,*Theor. Appl. Genet.* 87:757 (1994)). For the *Ty-1* allele, Zamir *et al.* found the restriction fragment length polymorphism (RFLP) TG97, which was originally mapped by Tanskley (Tanskley *et al.*, *Genetics* 132:1141 (1992)) to be tightly linked to the *Ty* locus. Similarly, Williamson *et al.* found the REX-1 locus to be tightly associated with the *Mi* locus.

As mentioned previously, any type of molecular assay can be used to perform the marker-assisted selection of the present invention. Such molecular assays are well known to those skilled in the art. For example, the TG97 clone and the REX-1 genomic regions were sequenced using techniques well known in the art in order to determine the kind of polymorphisms present between *L. esculentum* and *L. chilense* at the TG97 locus and between *L. esculentum*, *L. chilense* and *L. peruvianum* at the REX-1 locus.

As discussed briefly previously, one type of marker-assisted selection technique is a CAPS assay. By way of example, but not of limitation, for use in the methods of the present invention, a CAPS assay can be developed for the purpose of distinguishing between the *Ty*+ allele from *L. esculentum* and the *Ty-1* allele from *L. chilense*. Such an assay can be used to introgress the *Ty-1* allele into *L. esculentum* in the coupling phase with an allele (such as the *Mi-1* allele from *L. peruvianum*) that encodes for resistance to root-knot nematodes.

More specifically, SEQ ID NO.: 10 provides the polynucleotide sequence near the *Ty-1* allele from *L. chilense* LA1969. SEQ ID NO.: 11 provides the polynucleotide sequences near the wild type *Ty*+ allele from *L. esculentum* at the TG97 locus. Figure 5 provides a sequence comparison of these two polynucleotide sequences (SEQ ID NOS: 10 and 11). In this figure, 19 single nucleotide polymorphisms, or SNPs, are highlighted. SNPs can be substitutional mutants, insertions or deletions, which are commonly called INDELS. Using the sequence comparison in Figure 5 and the polymorphisms between

the alleles, those skilled in the art recognize that any number of marker-assisted assays and primers can be developed for the 19 SNPs. Such an assay can be used to distinguish between the resistant *Ty-1* allele from the susceptible *Ty+* allele.

More specifically, one skilled in the art could examine the polymorphic regions of the polynucleotide sequences shown in Figure 5 (SEQ ID NOS.: 10 and 11) and identify the polymorphisms between these polynucleotides sequences, and develop a molecular test to distinguish any of the polymorphisms. By way of example, and by no means limiting, one skilled in the art could exploit the two adjacent polymorphisms found at base pair positions 97-98 in the *L. chilense* sequence (*Ty-1* – SEQ ID NO.: 10*)* and at positions 96-97 in the *L. esculentum* sequence (*Ty+* - SEQ ID NO.: 11). As a consequence of these two adjacent SNP polymorphisms, there is a difference in a restriction endonuclease recognition site. The enzyme *Taq*I recognizes and cuts DNA within the sequence TCGA, which is found at base pair positions 95-98 in the *L. chilense* sequence (*Ty-1*). Because of the two adjacent polymorphisms, there is no *Taq*I restriction site at the corresponding positions in the *L. esculentum* sequence (*Ty+*). Examination of the rest of the sequences reveals no other *Taq*I restriction sites; therefore, these SNPs are good candidates for designing a CAPS assay. To design a CAPS assay around this restriction site polymorphism, sequences are entered into a primer design software program (such as "Primer Design" available from DNAStar, Madison, WI, or, "Primer Express" from Applied Biosystems, Foster City, CA, or "Primer 3" from Whitehead Institute, Cambridge, MA), which are known in the art. Once the primers have been designed and synthesized, then PCR of the genomic DNA extracted from the tomato of interest is conducted. The conditions for conducting PCR are well known in the art and can be found in J. F. Burke editor *PCR: Essential Techniques.* John Wiley and Sons. (1996). After the PCR reaction, the amplified product or products are exposed to the *Taq*1 restriction enzyme (commercially available from New England Biolabs, Beverly, MA). Because of the tight linkage of TG97 and the *Ty* locus, this TG97-based PCR test can predict the alleles present at the nearby *Ty* locus.

Using these assay conditions and primer choice options known to those skilled in the art, these software programs can be used to design one or more primers to amplify a region of tomato genomic DNA that includes the polymorphic region to be measured. Preferably, the position of the SNP(s) relative to the position of the primers should

enhance the resolution and subsequent scoring of the DNA fragments. In this example, the primers will amplify a fragment of approximately 398 base pairs. The position of the polymorphic restriction site is preferably asymmetrically located within the amplicon; the *Taq*I restriction site polymorphism in this example is 95 base pairs from the 5' end of the sequence 5' TAATCCGCTGTTACCTCTCC TT 3' (SEQ ID NO.: 1), and 303 base pairs from the 5' end of the sequence 5' CGGATGACTT CAATAGCAAT GA 3' (SEQ ID NO.: 2).

Implementing the above described marker-assisted selection example assay begins with selecting a tomato plant or plants that one wishes to determine the allelic composition at the *Ty* locus. Genomic DNA is then isolated individually from the sample(s). An aliquot of this DNA is used with the assay primers: TAATCCGCTGTTACCTCTCC TT 3' (SEQ ID NO.: 1) and 5' CGGATGACTT CAATAGCAAT GA 3' (SEQ ID NO.: 2) to amplify a 398 base pair DNA amplicon using standard PCR techniques.

After the PCR reaction, the amplified product or products are exposed to the *Taq*1 restriction enzyme (commercially available from New England Biolabs, Beverly, MA). Because of the tight genetic linkage between TG97 and the *Ty* locus, this TG97-based PCR test can predict the allelic combination present at the nearby *Ty* locus.

Once the amplified DNA has been digested with the restriction enzyme for a sufficient amount of time (about 2 to about 3 hours), the reaction is subjected to gel electrophoresis for resolution of the fragments. Techniques for performing gel electrophoresis are well known to those skilled in the art. As discussed above, if the *Ty-1* allele is present, the DNA amplified in the PCR test will be digested into two fragments of 95 and 303 base pairs. A plant that contains the susceptible Ty^+ allele in its genome exhibits a single DNA fragment in the gel of approximately about 398 base pairs using this test (See Figure 3).

A plant that contains the *Ty-1* allele in a heterozygous condition in its genome (*Ty-1*/Ty^+) exhibits all three of the above-described DNA fragments in the gel. Plants identified pursuant to this example assay containing the *Ty-1* allele in its genome in a homozygous or heterozygous condition can be selected for further molecular analysis

and/or breeding. An example of how this assay can be conducted is shown in the PCR Assay in Example 2.

This method of introgressing superior resistance alleles into tomato can be iterative. For example, when plant breeders or plant pathologists discover another polymorphic variant from an wild relative accession of tomato, like *Lycopersicon chilense, Lycopersicon peruvianum, Lycopersicon cerasiforme, Lycopersicon pimpinellifolium, Lycopersicon cheesmanii, Lycopersicon parviflorum, Lycopersicon chmielewskii, Lycopersicon hirsutum, Lycopersicon pennellii* and *Solanum lycopersicoides* that provided higher levels of pathogen resistance to TYLCV, or lower genetic drag, those skilled in the art recognize that a new combination of most efficacious alleles at the *Ty* and *Mi* loci can be combined using the methodology described herein. To design a new molecular test to distinguish a new donor allele, those skilled in the art recognize that one would first determine the DNA sequence at the marker locus TG97, and then compare the sequence with the corresponding DNA from the recipient allele. With this DNA comparison, those skilled in the art could identify either new sequence polymophisms or whether existing polymorphisms previously uncovered with other comparisons remain. Using these data, one could either design a new molecular test, or use an existing test to facilitate the introgression any allelic combination at the *Ty* and *Mi* loci together *in cis*.

Similar methods as those described above can be used to introgress an allele that encodes for resistance to root-knot nematodes. By way of an example, but not of limitation, an assay for determining the presence of the *Mi-1* allele in the genome of a tomato plant will be described. An assay for determining the presence of the *Mi-1* allele can be determined in a manner similar to those used to identify the *Ty-1* allele described previously. However, if it is suspected that the plant under investigation might possibly contain the *Mi-J* allele, then the identification of the presence of the *Mi-1* allele in the genome of the plant may, but not necessarily, involve conducting two (2) molecular assays, such as those described below. Nonetheless, the order in which these assays are performed is not critical.

Based on the data published by Williamson *et al., Theoretical and Applied Genetics* 87:757-763 (1994), the polynucleotide sequences of *L. esculentum*, *L.*

peruvianum and *L. chilense* were determined at the locus nearby to *Mi* that is referred to as REX-1. These three polynucleotide sequences are provided in SEQ ID NOS.: 7 (*Mi+*), 8 (*Mi-1*) and 9 (*Mi-J*). A comparison of these polynucleotide sequences is shown in Figure 6, which reveals 21 SNPs between these three sequences.

By comparing these three polynucleotide sequences and identifying the polymorphisms, those skilled in the art recognize that any number of marker-assisted assays can be developed for the 21 SNPs, such as, but not limited to, a CAPS assay. To develop a CAPS assay that distinguishes between the resistant alleles *Mi-1* or *Mi-J* from *L. peruvianum and L. chilense,* and the *Mi+* allele from *L. esculentum*, one skilled in the art would first examine the polymorphic regions of the sequence comparisons as shown in Figure 6. For example, one skilled in the art could exploit the SNP found at position 603 in SEQ ID NOS.: 7, 8 and 9. This SNP causes a difference in whether the enzyme *Taq*I recognizes and digests the sequence TCGA found in the *L. chilense* (SEQ ID NO.: 8), nor the *L. peruvianum* (SEQ ID NO.: 9). Because of the polymorphism the *L. esculentum* SEQ ID NO.: 7 (positions 600-603) does not have this *Taq*I recognition site at positions 600-603. To design a CAPS assay around this restriction site polymorphism, sequences are entered into a primer design software program (such as "Primer Design" available from DNAStar, Madison, WI, or, "Primer Express" from Applied Biosystems, Foster City, CA, or "Primer 3" from Whitehead Institute, Cambridge, MA) that are known in the art. The conditions for conducting PCR are well known in the art and can be found in J. F. Burke editor *PCR: Essential Techniques*. John Wiley and Sons. (1996). After the PCR reaction, the amplified product is exposed to the *Taq*1 restriction enzyme (commercially available from New England Biolabs, Beverly, MA). Because of the tight linkage of REX1 and the *Mi* locus, this REX1-based PCR test can distinguish the resistant alleles from the susceptible allele found in *L. esculentum* present at the nearby *Mi* locus.

Using the assay conditions and the primer choice options known to those skilled in the art, these software programs can aid in the choice of primer design to amplify a region of tomato genomic DNA that includes the polymorphic region to be measured. Preferably, the position of the SNP(s) relative to the position of the primers should enhance the resolution and subsequent scoring of the DNA fragments. In this example, the primers will amplify a fragment of approximately 595 base pairs. The position of the

polymorphic restriction site is preferably asymmetrically located; the *Taq*I restriction site polymorphism in this example is 450 base pairs from the 5' end of the sequence 5' AACCGTGGAC TTTGCTTTGA CT 3' (SEQ ID NO.: 3) and 145 base pairs from the 5' end of the sequence 5' TAAGAACAGG GACTCAGAGG ATGA 3' (SEQ ID NO.: 4).

Implementing the above described marker-assisted selection example assay begins with selecting a tomato plant or plants that one wishes to determine the allelic composition at the *Mi* locus. Genomic DNA is then isolated individually from the sample(s). An aliquot of this DNA is used with the example assay primers AACCGTGGAC TTTGCTTTGA CT 3' (SEQ ID NO.: 3) 5' TAAGAACAGG GACTCAGAGG ATGA 3' (SEQ ID NO.: 4) to amplify a 595 base pair DNA amplicon using standard PCR techniques.

After the PCR reaction, the amplified product is exposed to the *Taq*1 restriction enzyme (commercially available from New England Biolabs, Beverly, MA). Because of the tight genetic linkage between REX-1 and the *Mi* locus, this PCR test can predict the allelic combination present at the nearby *Mi* locus.

Once the amplified DNA has been digested with the restriction enzyme for a sufficient amount of time (about 2 to about 3 hours), the reaction is subjected to gel electrophoresis for resolution of the fragments. Techniques for performing gel electrophoresis are well known to those skilled in the art. If the *Mi-1* or *Mi-J* alleles are present in the amplified and digested DNA, two fragments or approximately 145 and approximately 450 base pairs will be observed. A plant containing the *Mi*+ allele will produce a single DNA fragment after PCR and enzyme digestion of approximately 595 base pairs (See Figure 3).

A plant that contains the *Mi-1* allele or *Mi-J* allele in its genome in a heterozygous condition with the *Mi*+ allele exhibits all three of the above-described DNA fragments. Plants identified pursuant to this assay as containing the *Mi-1* allele in a homozygous or heterozygous condition can be selected for further molecular analysis and breeding.

By examining the SNP polymorphisms in Figure 6, one skilled in the art could design a marker-assisted selection assay to distinguish the *Mi-J* allele from either the *Mi-*

1 or *Mi+* alleles. For example, one skilled in the art could exploit the polymorphism found at position 754 in Figure 6 and develop a marker-assisted selection assay. This polymorphism is within the sequence recognition sequence of the restriction endonuclease *Nla*III. This enzyme has a recognition sequence of CATG, which is found in the *L. chilense* sequence between positions 752 and 755 (Figure 6). By designing primers that encompass this area within the PCR amplicon, one skilled in the art can design a CAPS assay that will be able to distinguish the presence of *L. chilense* DNA from either the *L. peruvianum* or *L. esculentum* DNAs.

To design a CAPS assay around this restriction site polymorphism, sequences are entered into a primer design software program (such as "Primer Design" available from DNAStar, Madison, WI, or, "Primer Express" from Applied Biosystems, Foster City, CA, or "Primer 3" from Whitehead Institute, Cambridge, MA) that are known in the art. The conditions for conducting PCR are well known in the art and can be found in J. F. Burke editor *PCR: Essential Techniques*. John Wiley and Sons. (1996). After the PCR reaction, the amplified product or products are exposed to the *Taq*1 restriction enzyme (commercially available from New England Biolabs, Beverly, MA). Because of the tight linkage of REX1 and the *Mi* locus, this REX1-based PCR test can distinguish the *Mi-J* allele from either the susceptible allele or the *Mi-1* allele present at the nearby *Mi* locus.

Using the assay conditions and the primer choice options known to those skilled in the art, these software programs can aid in the choice of primer design to amplify a region of tomato genomic DNA that includes the polymorphic region to be measured. Preferably, the position of the SNP(s) relative to the position of the primers should enhance the resolution and subsequent scoring of the DNA fragments. In this example, the primers will amplify a fragment of approximately 282 base pairs.

The position of the polymorphic restriction site is preferably asymmetrically located; the *Nla*III restriction site polymorphism in this example is 158 base pairs from the 5' end of the sequence 5' CTACGGAGGA TGCAAATAGA A 3' (SEQ ID NO.: 5) and 124 base pairs from the 5' end of the sequence 5' AATCATTATT GTCACACTTC CCC 3' (SEQ ID NO.: 6).

Implementing the above described marker-assisted selection example assay begins with selecting a tomato plant or plants that one wishes to determine the allelic composition at the *Mi* locus. Genomic DNA is then isolated individually from the sample(s). An aliquot of this DNA is used with the example assay primers 5' CTACGGAGGA TGCAAATAGA A 3' (SEQ ID NO.: 5) and 124 base pairs from the 5' end of the sequence 5' AATCATTATT GTCACACTTC CCC 3' (SEQ ID NO.: 6) to amplify a 595 base pair DNA amplicon using standard PCR techniques.

Once the amplified DNA is obtained, it is subjected to the *NlaIII* restriction enzyme for a sufficient amount of time (about 2 to about 3 hours), it is subjected to gel electrophoresis for resolution of the fragments. If the *Mi-J* allele is present in the amplified DNA, the *NlaIII* enzyme will digest the amplified DNA into two fragments of approximately about 124 and approximately about 158 base pairs. A plant that contains the *Mi-1* allele from *L. peruvianum* (or the wild type allele from *L. esculentum)* in its genome exhibits a single DNA fragment in the gel of approximately about 282 base pairs (See Figure 4). A plant that contains the *Mi-J* allele in its genome in a heterozygous condition together with either the *Mi-1* or Mi^{+} allele exhibits all three of the above-described DNA fragments in the gel. Plants identified pursuant to this assay as containing the *Mi-1* allele in a homozygous condition together with the *Ty*-1 allele can be selected for further molecular analysis and/or breeding. An example of how this assay can be conducted is shown in PCR Assay #2 in Example 4.

The above-described assays can be used individually and collectively in a breeding program to facilitate the breeding and/or selection of tomato plants that contain the *Ty-1* allele and the *Mi-1* allele in the coupling phase. One example of how these methods can be used is described below. For example, a first inbred tomato line may be crossed with a second inbred tomato line to produce a hybrid plant. The first tomato plant used in the cross can contain the *Ty-1* allele and the second plant the *Mi-1* allele in its genome, or vice versa (See Figure 1). A resulting plant (F_1 hybrid) is then allowed to self-pollinate, fertilize and set seed (F_2 seed). The F_2 plants are grown from the F_2 seed. These plants are then subjected to DNA extraction, the techniques of which are known in the art (See Hnetkovsky *et al.*, *Crop Sci.,* 36 (2): 393-400 (1996)). Once the DNA extraction is complete, the above-described assays can be used to identify F_2 plants that contain the *Ty-1* in a heterozygous state and *Mi-1* allele in a homozygous state.

Alternatively, one can identify an F2 plant that contains the *Ty-1* allele in a homozygous state and the *Mi-1* allele in the heterozygous state. Because recombination between the *Ty* and *Mi* loci is low, finding these recombinants in the F2 is rare. Theoretically, one could find plants with the *Ty-1* and *Mi-1* alleles both fixed in the homozygous state, but the probability of finding such a plant is the approximate product of the probability of finding the individual recombinants. Thus, in a theoretical example, if the probability of finding each individual recombinant is 1 in 1000, the probability of finding a rare fixed recombinant would be 1 in 1,000,000. The assays described herein can also be used to determine if the *Ty-1* and/or *Mi-1* alleles are present in the genome of the plant in a homozygous or heterozygous condition. Depending upon the results of the assay(s), further breeding and molecular characterization may be necessary. For example, if the goal of the breeding program is to create an inbred line and the results of one or more of the above-described assays for a specific tomato plant being tested reveal that the plant contains the *Mi-1* allele in its genome in a homozygous condition and the *Ty-1* allele in a heterozygous condition, then that plant may be subjected to further self-fertilization, breeding and molecular characterization using one or more of the assays described herein until it has been determined that said plant and its progeny, after selfing, contains both the *Mi-1* allele and the *Ty-1* allele in its genome in a homozygous condition. Once the *Mi-1* and *Ty-1* alleles are created in the coupling phase, or *in cis*, they will be inherited together. This heritable block of multiple resistance genes provides the plant breeder with flexibility in creating new hybrids, while also allowing the plant breeder the ability to mask the genetic drag effects of the wild species introgressions with the second inbred parent.

As mentioned briefly above, the methods of the present invention can be used to create new and superior inbred lines. These inbred lines can be used in subsequent breeding to create hybrid tomato plants that are resistant to TYLCV and root-knot nematodes and also possess other commercially desirable characteristics. Such inbred lines are useful in breeding because these lines allow for the transfer of the *Ty-1* and *Mi-1* alleles as a single coinheritable unit that facilitates rapid breeding. Moreover, the above-described methods are also useful in confirming that an inbred line does in fact contain the *Ty-1* allele and the *Mi-1* allele in its genome in a homozygous condition and is maintaining its homozygosity. Once this confirmation is obtained, the inbred line is can be used in crosses with a second inbred line to transfer the *Ty-1* allele and *Mi-1*

allele to a hybrid tomato plant as a single coinheritable unit. The second inbred line can carry the wild type alleles *Ty*$^+$ and *Mi*$^+$ to mask the effects of genetic drag.

By way of example, and not of limitation, examples of the present invention will now be given.

Example 1: Protocol for Tomato DNA Extraction

The following protocol can be used to extract DNA from tomato germplasm.

1. Collect a plant part that is approximately the size of a well in the 96 well microtiter plate format. Preferably, either a seed sample or a tissue sample is taken from young leaves .

2. Add 150 µl extraction buffer (200 mM Tris-HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS) to the sample and macerate the tissue.

3. Centrifuge the plate for 15 minutes at 1900-x g at 15°C.

4. Transfer 100 µl of the supernatant fraction to a new 96 well plate that contains 100 µl of 2.5M potassium acetate (pH 6.5) in each well. Mix by shaking for approximately 2 minutes at 200 rpm.

5. Centrifuge the plate for 15 minutes at 1900-x g at 15°C.

6. Transfer 75 µl of the supernatant fraction to a new 96 well plate containing 75 µl isopropanol. Mix, then shake for 2 minutes at 200 rpm.

7. Centrifuge the plate for 15 minutes at 1900-x g at 15°C.

8. Remove supernatant fraction and add 200 µl 70% ethanol to the pellet fraction. Shake at 200 rpm for 5 minutes, and then incubate overnight at -20°C.

9. Centrifuge the plate for 15 minutes at 1900-x g at 15°C.

10. Remove supernatant fraction. Add 200 ul of 70% ethanol to the pellet, allowing the alcohol to wash the pellet for 1 hour at room temperature.

11. Centrifuge the plate for 15 minutes at 1900-x g at 15°C.

12. Discard the supernatant fraction and dry the pellet fraction at room temperature (this takes about 1 hour).

13. Dissolve the pellet fraction in 100 µl TE (10 mM Tris, pH 8.0, 1mM EDTA, 5 µg/ml RNAase A) for 15 minutes at 37°C. Unless proceeding to the PCR step, the DNA can be stored at 4°C or - 20°C.

Example 2: PCR Assay for Identifying the Alleles Present at the *Ty* Locus in Tomato Germplasm

The following assay can be used to identify the allelic composition at the *Ty* locus in tomato plants using DNA extracted from said leaf tissue pursuant to Example 1.

A 25 microliter PCR reaction is prepared with the following components:

Ten picomoles of each of the following primers: 5' TAATCCG TCG TTACCTCTCC TT 3' (SEQ ID NO.:1) and 5' CGGATGACTT CAATAGCAAT GA 3' (SEQ ID NO.:2).

A final concentration of 200 µM of each deoxyribonucleoside-5'-triphosphates (dNTPs) (available from Pharmacia, Kalamazoo, MI or Invitrogen, Carlsbad, CA).

2.5 uL of 10 X PCR buffer (available from Applied Biosystems, Forest City, CA)

1.25 units *Taq* polymerase (available from Applied Biosystems)

1 uL of DNA preparation

water up to about 25 microliters.

The PCR reaction is performed in an Applied Biosystems, 96-well GeneampR PCR system 9700 (Applied Biosystems, Foster City, CA) using the following reaction parameters:

1. An initial denaturation step of 2 minutes at 94°C.

2. Thirty-five cycles with each cycle having 3 steps. These steps are: a) 30 seconds at 92°C; b) 30 seconds at 50°C; and c) 90 seconds at 72° C.

3. Five minutes at 72°C.

Following PCR, a restriction enzyme digest is prepared as follows.

To the PCR reaction, add:

3 uL microliters of 10X restriction enzyme buffer (commercially available from New England Biolabs, Beverly, MA).

0.25 microliters of *TaqI* restriction enzyme (commercially available from New England Biolabs, Beverely, MA)

1.75 microliters of water.

Incubate at 65°C for 3 hours.

Following the enzyme digest, a loading buffer is added and the samples are loaded onto a 1.5% agarose gel and electrophoresed for several hours through a Tris/Borate/EDTA buffer. Varying loading buffers are known to those skilled in the art. The purpose is to add chemicals that will make the loaded samples sink into the wells, and to have a dye that allows one to monitor the progress of the electrophoresis visually. In addition to the PCR samples, at least one lane of molecular mass standards is also loaded.

Following the electrophoresis, DNA fragments are stained with Ethidium Bromide (Sigma Chemical Company, St. Louis, MO) under conditions well known to those skilled in the art and visualized by exciting the stained DNA with ultraviolet light.

Presence of the *Ty-1* allele in the plant can be predicted by the observation of DNA fragments of approximately 95 base pairs and about 303 base pairs in length. The presence of the susceptible Ty^+ allele in the plant can be predicted by the observation of a DNA fragment in the gel of approximately about 398 base pairs. Plants heterozygous of the *Ty-1* allele can be predicted by the observation of DNA fragments of all three sizes (95, 303 and 398 base pairs).

Example 3: PCR Assay #1 for Identifying the alleles at the Mi locus in Tomato Germplasm

The following assay can be used to identify the presence of the *Mi-1* or *Mi-J or* Mi^{+} alleles in a tomato plant using DNA extracted from said germplasm pursuant to Example 1.

A 25 microliter PCR reaction is prepared with the following components:

Ten picomoles of each of the following primers: 5' AACCGTGGAC TTTGCTTTGA CT 3' (SEQ ID NO.:3) and 5' TAAGAACAGG GACTCAGAGG ATGA 3' (SEQ ID NO.:4).

A final concentration of 200 µM of each deoxyribonucleoside-5'-triphosphates (dNTPs) (available from Pharmacia, Kalamazoo, MI or Invitrogen, Carlsbad, CA)

2.5 uL of 10 X PCR buffer (available from Applied Biosystems, Forest City, CA)

1.25 units of *Taq* polymerase (available from Applied Biosystems)

1 uL of tomato DNA preparation

water up to about 25 microliters.

The PCR reaction is performed in an Applied Biosystems, 96-well GeneampR PCR system 9700 (Applied Biosystems), using the following reaction parameters:

1. An initial denaturation step at 2 minutes at 94°C.
2. Thirty-five cycles with each cycle having 3 steps. These steps are: a) 30 seconds at 92°; b) 30 seconds at 50°C; and c) 90 seconds at 72°C.
3. Five minutes at 72°C.

Following PCR, a restriction enzyme digest is prepared as follows.

To the PCR reaction, add:

3 uL microliters of 10X restriction enzyme buffer (commercially available from New England Biolabs, Beverly, MA).

0.25 microliters of *TaqI* restriction enzyme (commercially available from New England Biolabs, Beverely, MA)

1.75 microliters of water.

Incubate at 65°C for 3 hours.

Following the enzyme digest, a loading buffer is added and the samples are loaded onto a 1.5% agarose gel and electrophoresed for several hours through a Tris/Borate/EDTA buffer. In addition to the PCR samples, at least one lane of molecular mass standards is also loaded.

Following the electrophoresis, DNA fragments are stained with Ethidium Bromide and visualized by exciting the stained DNA with ultraviolet light.

Presence of the *Mi-1* or *Mi-J* allele in the plant can be predicted by the observation of DNA fragments of approximately 145 base pairs and about 450 base pairs in length. Presence of the Mi^+ allele in the plant can be predicted by the observation of a DNA fragment of approximately 595 base pairs. A heterozygous genotype of either *Mi-1*/Mi^+ or *Mi-J*/Mi^+ is predicted by the presence of all three of the above-described DNA fragments in the gel.

Example 4: PCR Assay #2 for Identifying the *Mi-1* or *Mi-J* Alleles in Tomato Germplasm

The following assay can be used to identify the presence of the *Mi-1* allele in tomato germplasm using DNA extracted from said germplasm pursuant to Example 1.

A 25 microliter PCR reaction is prepared with the following components:

Ten picomoles of each of the following primers: 5' CTACGGAGGA TGCAAATAGA A 3' (SEQ ID NO.:5) and 5' AATCATTATT GTCACACTTC CCC 3' (SEQ ID NO.:6)

A final concentration of 200 µM of each deoxyribonucleoside-5'-triphosphates (dNTPs) (available from Pharmacia, Kalamazoo, MI or Invitrogen, Carlsbad, CA)

2.5 uL of 10 X PCR buffer (available from Applied Biosystems, Forest City, CA)

1.25 units of *Taq* polymerase (available from Applied Biosystems)

1 uL of tomato DNA preparation

water up to about 25 microliters.

The PCR reaction is performed in an Applied Biosystems, 96-well GeneampR PCR system 9700 (Applied Biosystems, Foster City, CA), using the following reaction parameters:

1. An initial denaturation step at 2 minutes at 94°C.
2. Thirty-five cycles with each cycle having 3 steps. These steps are: a) 30 seconds at 92°C; b) 30 seconds at 45°C; and c) 90 seconds at 72°C.
3. Five minutes at 72°C.

Following PCR, a restriction enzyme digest is prepared as follows.

To the PCR reaction, add:

3.5 uL microliters of 10X restriction enzyme buffer.

0.25 microliters of *NlaIII* restriction enzyme (commercially available from New England Biolabs)

6.25 microliters of water.

Incubate at 37°C for 3 hours.

Following the enzyme digest, a loading buffer is added and the samples are loaded onto a 1.5% agarose gel using a Tris/Borate/EDTA buffer, and electrophoresed for several hours. In addition to the PCR samples, at least one lane of molecular mass standards is also loaded on the gel.

Following the electrophoresis, DNA fragments are stained with Ethidium Bromide and visualized by exciting the stained DNA with ultraviolet light.

Presence of the *Mi-J* allele in the plant can be predicted by the observation of DNA fragments of approximately 124 base pairs and about 158 base pairs in length. Presence of either the *Mi-1* allele or the wild type allele from *L. esculentum* in the plant can be predicted by the observation of a single DNA fragment in the gel of approximately 282 base pairs. The presence of the *Mi-J* allele in a heterozygous condition is predicted by the observation of all three of the above-described DNA fragments in the gel.

Example 5: Protocol for Determining Resistance to *Meloidogyne incognita*

A pathology test for measuring *Meloidogyne incognita* resistance is based on a rating of the amount of root galls. A variety is considered resistant when the average

rating is between zero (0) and one (1), intermediately resistant when the average rating is between one (1) and two (2) and susceptible when the average rating is near three (3) and above.

Approximately thirty (30) seedlings per line are tested for resistance to *Meloidogyne incognita* in greenhouse benches filled with a mixture of peat, vermiculite and sand in a ratio of 4:1:1. At the same date of sowing the tomato seeds, pieces of tomato root tissue infected with *M. incognita* (incubated for two (2) months: roots are showing mature egg masses) are placed in holes between the rows. Plants are incubated in the greenhouse at temperatures between 22 and 26 degrees centigrade (°C). The rating is done 28 days after sowing by pulling up each plant and inspecting the roots for the presence of galls. The following rating scale is used:

Rating score	Severity of symptoms
0	No galls present
1	One (1) or two (2) small galls
2	Some galls, small size, disseminated
3	Several galls, bigger in size, disseminated
4	Many galls, in chains, deformed roots

Example 6: Protocol for Determining Resistance to Tomato Yellow Leaf Curl Virus (TYLCV)

This example describes a protocol for determining whether tomato plants are resistant, intermediate resistant or susceptible to TYLCV.

Plants are grown in a field with natural infection of TYLCV through *Bemisia tabaci*. Naturally occurring field infection is a preferred method of determining resistance in areas where the virus is endemic, as the movement of the viral pathogen can be controlled by various governmental agencies (quarantine disease). For example, the United States Department of Agriculture will not normally allow the introduction of the TYLCV pathogen into most tomato growing regions of the United States where the pathogen does not normally exist. Conducting disease screens under controlled conditions is cumbersome because of the need to raise the insects (*Bemisia tabaci)* for the transmission of the virus.

The following scale (0 to 4) was used to score for disease symptoms of TYLCV:

Rating score	Severity of symptoms
0	no symptoms
1	slight yellowing of leaves
2	clear yellowing symptoms on leaves with leaf curl
3	Stunted plants with severe symptoms of yellowing of leaves and leaf curl
4	Severely stunted plants with small yellowing curled leaves

A variety is rated as resistant to TYLCV when the score is 0-1, intermediate resistant when the score is 2 and susceptible when the score is either 3 or 4.

Alternatively, protocols for determining TYLCV that are known in the art can also be used. "Tomato Yellow Leaf Curl Virus from Sardinia is a whitefly-transmitted monopartite geminivirus"; A. Keyyr-Pour, M. Bendahmane, V. Matzeit , G.P. Acotto, S. Crespi, B. Gronenborn.; Nucleic Acids Research, Volume 19, p. 6763-6769.; Tomato Yellow Leaf Curl Virus: a whitefly transmitted geminivirus with a single genomic component" , N. Navot, E. Pichersky, M. Zeidan, D. Zamir, H. Czosnek. Virology, 185, 1991, p. 151-161.

Example 7: Illustration of breeding program and development of Tomato Plants Containing the *Ty-1* Allele and the *Mi-1* Allele in The Coupling Phase

In the fall of 1997, in Nîmes, France, a cross was made between *L. esculentum* line FIR 16-176 and *L. esculentum* line FDR 16-2045 (which is the subject of U.S. Patent No. 6,414,226) resulting in the hybrid number 1652817. FIR 16-176 is a proprietary line of Seminis Vegetable Seeds, Inc., the assignee of the present invention. FIR 16-176 contains the *Mi-1* allele from *L. peruvianum*. FDR 16-2045 contains the *Ty-1* gene from *L. chilense* and the *Mi-J* allele from *L. chilense*.

In the summer of 1998, in Nîmes, France, selfed seed of hybrid 1652817 was harvested in bulk (F_2 seed). In January 2000 in Nîmes, France, the F_2 seed was planted and allowed to grow into plants. Leaf samples of 504 of the resulting F2 plants were tested. A leaf or a piece of a leaf was taken from each of these plants and DNA extracted pursuant to Example 1. Next, the assay described in Example 2 was performed. A total of 377 plants were identified pursuant to the assay in Example 2 as being homozygous or heterozygous for the *Ty-1* allele. These plants were selected.

Next, the assays described in Examples 3 and 4 were conducted on the selected 377 plants in order to distinguish plants containing the *Mi-1* allele from those containing the *Mi-J* allele. Any plant homozygous for *Mi-1* and heterozygous for *Ty*$^+$/*Ty-1* and any plant homozygous for *Ty-1* and heterozygous for *Mi-1*/*Mi-J* would have arisen as a result of a recombination event or crossover between the *Ty* and the *Mi* loci. Selfing of either of such recombinant plants allows one to screen the F3 for fixation of both traits *Ty-1* and *Mi-1*.

A total of eight (8) such recombinant plants were identified. Two (2) plants out of these eight (8) were selected. These two (2) plants were selected visually as having the most favorable horticultural phenotype. These two plants were identified as plant number "20" and plant number "251". Both of these plants were heterozygous for the *Ty-1* allele and homozygous for the *Mi-1* allele. The genotypic presentation of these plants is shown in Figure 1 and below:

$$\frac{Ty\text{-}1 \quad Mi\text{-}1}{Ty^+ \quad Mi\text{-}1}$$

In the spring of 2000, in Nîmes, France, plant number "20" and plant number "251" were planted in pots and allowed to self-pollinate. The resulting F_3 seeds from each of these plants were harvested separately. The F_3 seed was coded 97.5281.M.20 and 97.5281.M.251. The F_3 seeds were planted in pots in the glasshouse in Nîmes, France and allowed to grow into plants. Of each of the two (2) selfed progenies 40 F_3 plants were selected for further DNA testing. A leaf or a piece of a leaf was taken from each of these plants and the DNA extracted pursuant to Example 1. Next, the assays described in Examples 2, 3 and 4 were performed. Based on the results of these assays, four (4) plants from 97. 5281.M.20 and three (3) plants of 97.5281.M.251 were

selected. These plants were selected because each had the *Ty-1* allele and the *Mi-1* allele in a homozygous condition, as shown below:

$$\frac{Ty\text{-}1 \quad Mi\text{-}1}{Ty^{+} \quad Mi\text{-}1}$$

Moreover, these plants had the most favorable horticultural phenotypes. As all of these plants are genetically homozygous for the *Ty-1/Mi-1* genotype, their progeny plants are expected to remain fixed, regardless of the crosses being made. This is due to the strong genetic linkage and repression of recombination between the *Ty* and the *Mi* loci.

In the fall of 2000, in Nîmes, France, the above selected seven (7) plants were allowed to self-pollinate and the resulting F_4 seed individually harvested. In January 2001, in Nîmes, France, the F_4 seed was planted and allowed to grow into plants. Of each of the seven progenies seven (7) plants, a total 49 plants, were selected for further testing. A leaf or a piece of a leaf was taken from each of these plants and the DNA extracted pursuant to Example 1. Next, the assays described in Examples 2, 3 and 4 were performed in order to confirm that the *Ty-1* allele and *Mi-1* allele were still present in a homozygous condition and that the *Mi-J* allele was absent. The results confirmed that the *Ty-1* allele and the *Mi-1* allele were each present in a homozygous condition in each of the 49 plants. The presence of the *Mi-J* allele was not detected.

In the spring of 2001, in Antalya, Turkey, a bulk mix of seed harvested from four (4) selected progenies of 97.5281.M.20 were planted in a plot identified as "486" and identified with code 97.5281.M.20.M. A bulk mix of seed from the three (3) selections of 97.5281.M. 251 was planted in a plot identified as "487" and identified with code 97.5281.M.251.M. The seed in each of the plots was allowed to grow into mature plants. In the plot identified as "486", one (1) plant was selected for further crossing and breeding, based on agronomic evaluation. In the plot identified as "487", two (2) plants were selected also based on agronomic evaluation. A leaf or a piece of a leaf was taken from each of these plants and the DNA extracted pursuant to Example 1. Next, the assays described in Examples 2, 3 and 4 were performed in order to reconfirm that the *Ty-1* allele and *Mi-1* allele were each present in a homozygous condition and that the *Mi-J* allele was absent. The results confirmed that the *Ty-1* allele and the *Mi-1* allele were each present in a homozygous condition in each of the three plants. The presence of

the *Mi-J* allele was not detected. Each of the three plants was allowed to self-pollinate and the resulting F_5 seed collected and coded as 97.5281.M.20.M.1, 97.5281.M.251.M.1 and 97.5281.M.251.M.2.

In the fall of 2001, in Nîmes, France, seed of 97.5281.M.251.M.1 was planted and allowed to grow into plants. Plants grown from this seed were crossed with eight (8) proprietary inbred *L. esculentum* lines owned by Seminis Vegetable Seeds, Inc., the assignee of the present invention, to make eight (8) hybrids. The eight (8) proprietary inbred lines did not contain the *Ty-1* allele at the *Ty* locus nor the *Mi-1* allele at the *Mi* locus. The seed of the eight resulting hybrids was harvested, labeled and coded 01C040 to 01C047. Additionally, at this time, three (3) lines coded 97.5281.M.20.M.1, 97.5281.M.251.M.1 and 97.5281.M.251.M.2 were tested for nematode resistance in a climate-controlled room in Nîmes, France using the assay described in Example 5. All three lines were determined to be resistant to *Meloidogyne incognita*.

In the fall of 2001, seeds from the lines labeled and coded 97.5281.M.20.M.1, 97.5281.M.251.M.1 and 97.5281.M.251.M.2, were planted in Antalya, Turkey in plots identified as "1109", "1110" and "1111". Lines were scored for TYLCV resistance as described using the protocol of Example 6 and found to be resistant. No selections were made.

In the spring of 2002, seeds from the lines labeled and coded 97.5281.M.20.M.1, 97.5281.M.251.M.1 and 97.5281.M.251.M.2, were planted in Antalya, Turkey in plots identified as "260", "261" and "262". One plant was selected from each of these plots (in total three (3) plants) based on agronomic evaluation. A leaf or a piece of a leaf was taken from each of these plants and the DNA extracted pursuant to Example 1. Next, the assays described in Examples 2, 3 and 4 were performed in order to confirm that the *Ty-1* allele and *Mi-1* allele were each present in a homozygous condition and that the *Mi-J* allele was absent. The results confirmed that the *Ty-1* allele and the *Mi-1* allele were each present in a homozygous condition in each of the three (3) plants and that the *Mi-J* allele was absent. These plants were each allowed to self-pollinate and the resulting F_6 seeds were harvested and coded as 97.5281.M.20.M.1.1, 97.5281.M.251.M.1.1 and 97.5281.M.251.M.2.1. Plants from these three (3) lines were tested for nematode resistance. This reconfirms the difference in efficacy between the *Mi-J* and the *Mi-1*

genes, especially in heterozygous condition. The three (3) lines will be used for further breeding and testing in new hybrid combinations.

In the fall of 2002, the lines coded as, 97.5281.M.20.M.1.1, 97.5281.M.251.M.1.1 and 97.5281.M.251.M.2.1 were planted in Antalya, Turkey in plots identified as "1505", "1506" and "1507". Of the eight (8) proprietary hybrids mentioned previously, the seeds were and planted in the spring of 2002 in Antalya, Turkey, and three (3) have been selected. These were the hybrids coded as 01C044, 01C045 and 01C046. These hybrids were chosen for agronomic reasons. These hybrids, as well as the three F_6 lines were all scored for TYLCV as described using the protocol in Example 6 and found to be resistant.

Each of these lines (97.5281.M.20.M.1.1, 97.5281.M.251.M.1.1 and 97.5281.M.251.M.2.1) was allowed to self-pollinate so that bulk seed could be made in the fall of 2002 in Antalya, Turkey. The resulting F_7 seed was planted as bulk in the spring of 2003 in Antalya, Turkey in plots identified as "271", "272" and "273", allowed to grow into mature plants and self-pollinate. From lines labeled and coded 97.5281.M.20.M.1.1.M, 97.5281.M.251.M.1.1.M and 97.5281.M.251.M.2.1.M, two (2) plants per line were selected based on agronomic evaluation thus resulting in six (6) F_8 lines, two (2) per family, that were labeled and coded 97.5281.M.20.M.1.1.M.1, 97.5281.M.20.M.1.1.M.2, 97.5281.M.251.M.1.1.M.1, 97.5281.M.251.M.1.1.M.2, 97.5281.M.251.M.2.1.M.1 and 97.5281.M.251.M.2.1.M.2, respectively. These lines will also be used for further breeding and testing in new hybrid combinations.

Table 1 below shows the number of plants per class that were inoculated with *M. incognita* from inbred and hybrid lines that are homozygous and heterozygous for the *Mi-J* and *Mi-1* alleles. This test was conduced in a greenhouse in March and May 2003 in Nîmes, France, pursuant to the protocol described in Example 5. The results in Table 1 and Figure 8 demonstrate the difference in efficacy between the *Mi-J* and *Mi-1* alleles, particularly when these alleles are present in the heterozygous condition, such as in the hybrid plants. The hybrid 01C045 was not tested for nematode resistance because there was a shortage of seed of this variety. All the seed of this experimental hybrid was used for planting and selection in the spring 2002 trial in Antalya.

TABLE 1

			3/24/2003					5/13/2003					Total				
Pedigree code		Genotype	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Breeding Lines																	
97.5281.M. 20.M.1.1		*Ty-1/Ty-1 Mi-1/Mi-1*	10	1				15	6				25	7			
97.5281.M. 251.M.1.1		*Ty-1/Ty-1 Mi-1/Mi-1*	25					66					91				
97.5281.M. 251.M.2.1		*Ty-1/Ty-1 Mi-1/Mi-1*	27					53					80				
FDR 16-2045		*Ty-1/Ty-1 Mi-J/Mi-J*	59	13	1	1		47	8	6			106	21	7	1	
Hybrids																	
Super Red[1]		*Ty-1/Ty$^+$ Mi-J/Mi$^+$*	11	2	6	1		11	15	7	10	2	22	17	13	11	2
Sadiq[2]		*Ty-1/Ty$^+$ Mi-J/Mi$^+$*	20	5	2			16	13	6	27		36	18	8	27	
Margo[3]		*Ty-1/Ty$^+$ Mi-J/Mi$^+$*	6	3	8			15	26	9	13		21	29	17	13	
01C041		*Ty-1/Ty$^+$ Mi-1/Mi$^+$*	25					28	1				53	1			
01C043		*Ty-1/Ty$^+$ Mi-1/Mi$^+$*	23					30					53				
01C044		*Ty-1/Ty$^+$ Mi-1/Mi$^+$*	24					39					63				
01C046		*Ty-1/Ty$^+$ Mi-1/Mi$^+$*	23					45	2				68	2			
Controls																	
Marmande verte[4]		*Ty$^+$/Ty$^+$ Mi$^+$/Mi$^+$*			7	11	20				30	21			7	41	41
1047		*Ty$^+$/Ty$^+$ Mi-1/Mi-1*	28	2				27	1				55	3			
99T6		*Ty$^+$/Ty$^+$ Mi-1/Mi$^+$*	51		2	2		17	2				68	2	2		

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All abstracts, references, patents and published patent applications referred to herein are hereby incorporated by reference.

[1] Super Red is a commercially available *Lycopersicon esculentum* variety that is available in the Middle East and is sold by the Assignee of the present invention, Seminis Vegetable Seeds, Inc.
[2] Sadiq is a commercially available *Lycopersicon esculentum* variety that is available in the Middle East and is sold by the Assignee of the present invention, Seminis Vegetable Seeds, Inc.
[3] Margo is a commercially available *Lycopersicon esculentum* variety that is available in the Middle East and is sold by the Assignee of the present invention, Seminis Vegetable Seeds, Inc.
[4]Marmande Verte is an anthocyanin-less variety which is a mutant of the publicly known variety Marmande listed in the Community Variety Catalogue of the European Community (Publication Journal of the EU, C167A).

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof.

Changes can be made to the composition, operation and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention.